Conferences and Reviews

Blood-Brain Barrier and New Approaches to Brain Drug Delivery

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Morbidity caused by brain dysfunction affects more than 50 million persons in the United States. Although new neuropharmaceuticals have the potential for treating specific brain diseases, they may not effectively enter brain from blood. Safe strategies are needed for drug delivery through the brain capillary wall, which makes up the blood-brain barrier in vivo. Two of these strategies are reviewed, as are related new developments in the molecular and cell biology of the brain capillary endothelium. The production of chimeric peptides represents a physiologic-based strategy for drug delivery. It entails the covalent coupling of the neuropharmaceutical to a brain transport vector, allowing transportation through the blood-brain barrier. Another strategy is biochemical opening of the blood-brain barrier: intracarotid leukotriene infusion is a method for selectively increasing blood-brain barrier permeability in brain tumors without affecting barrier permeability in normal brain tissue.

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WILLIAM M. PARDRIDGE, MD*: The morbidity of cancer and heart disease is dwarfed by illness caused by disorders of the brain, which affect 50 to 100 million persons in the United States (Table 1). The number of disorders of the brain commonly seen in the practice of internal medicine may be expanded even further because such problems as peptic ulcer, hypertension, or irritable bowel syndrome may be amenable to treatment by direct drug action on the brain.[1] In the 1990s, the Decade of the Brain, the traditional trial and error approach to drug development and design is giving way to rational drug design based on three-dimensional models of receptor structure. For example, new drugs for treating depression, stroke, Alzheimer's disease, fever, or anxiety may include corticotrophin-releasing hormone antagonists, calcitonin gene-related peptide agonists, amyloid protease inhibitors, α-melanocyte-stimulating hormone agonists, or cholecystokinin receptor antagonists, respectively.[1] In future, the driving force of drug design may be the expanding base of neuropeptide receptor biology that has emerged from research.

Unfortunately, drugs developed from rational drug design based on considerations of receptor biology may not be effective neuropharmaceuticals if these drugs are not transportable across the brain capillary wall, which makes up the blood-brain barrier in vivo. For example, the first product of recombinant DNA technology that may be used for treating a neurologic disease is nerve growth factor for treating Alzheimer's disease.[2] Nerve growth factor is a large protein of approximately 25,000 molecular weight and is not known to cross the blood-brain barrier.[3] Although proposals have been made to administer nerve growth factor through intraventricular drug delivery,[4] this approach requires a neurosurgical procedure. Moreover, the two caveats of intraventricular drug delivery are: the drug is distributed only to the surface of the brain and not deep into brain parenchyma,[5] and the drug is readily distributed into the peripheral circulation owing to rapid efflux of drug from the ventricular cerebrospinal

TABLE 1.—*Common Brain Disorders in the United States**

Disorder	*No. of Affected Persons, in millions*
Migraine headache	25
Alcohol abuse	25
Anxiety or phobia	25
Sleep disorders	20
Depression or mania	12
Drug abuse	10
Obsessive-compulsive disorder	4
Alzheimer's disease	3
Schizophrenia	2
Stroke	2
Epilepsy	2
Human immunodeficiency virus infection	1.5
Parkinson's disease	.5

*From Pardridge.[1]

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ABBREVIATIONS USED IN TEXT

cDNA = complementary DNA
mRNA = messenger RNA
p^I = isoelectric point

fluid compartment to the bloodstream through the arachnoid granulations at the superior sagittal sinus.[6] Another traditional approach for drug delivery are liposomes.[7] Liposomes, however, are too large to traverse the blood-brain barrier and have not been proved to be effective neuropharmaceutical delivery vehicles.[1]

The importance of developing effective neuropharmaceuticals and the lack of efficacy of existing strategies for drug delivery to brain emphasize the need to develop new approaches to drug transport across the blood-brain barrier. Two approaches discussed in this review have emanated as practical applications of fundamental basic blood-brain barrier research. The first approach is using chimeric peptides as a vehicle for neuropharmaceutical delivery through the blood-brain barrier. The second is biochemical opening of the blood-brain barrier.

Chimeric Peptides

Chimeric peptides are formed when a nontransportable pharmaceutical peptide—for example, β-endorphin for the treatment of pain—is coupled to a blood-brain barrier transport vector.[8] The latter is a peptide or plasma protein that normally enjoys receptor-mediated or absorptive-mediated transcytosis through the blood-brain barrier. Examples of compounds that traverse the blood-brain barrier by receptor-mediated transcytosis include insulin,[9] transferrin,[10] a monoclonal antibody to surface epitopes of the transferrin receptor,[11] and possibly insulin-like growth factors.[12] Proteins that traverse the blood-brain barrier via absorptive-mediated transcytosis include polycationic proteins, such as histone,[13] cationized albumin,[14] cationized antibodies,[15] or lectins, such as wheat germ agglutinin.[16] Although native albumin, which is an acidic protein with an isoelectric point (p^I) of 4 to 5, does not normally cross the blood-brain barrier, chemical modification of the protein, which results in the formation of cationized albumin, $p^I = 8$ to 9, causes the protein to undergo absorptive-mediated transcytosis through the blood-brain barrier.[14,17] More recent studies have shown that an antitransferrin receptor antibody[11] undergoes receptor-mediated transcytosis through the blood-brain barrier at rates that are severalfold faster than the transport of cationized albumin.[18]

Proteins such as cationized albumin or antitransferrin receptor monoclonal antibodies may be used as a vector to ferry across the blood-brain barrier compounds that are pharmacologically active in brain (Figure 1).[19] The four steps of the chimeric peptide transport process are receptor-mediated endocytosis at the blood side of the blood-brain barrier; receptor-mediated exocytosis of the chimeric peptide at the brain side of the blood-brain barrier with release into brain interstitial fluid; thiol-based cleavage of the disulfide bond adjoining the chimeric peptide, which results in the release within brain of pharmacologically active peptide; and binding of the pharmacologically active peptide to its cognate receptor in brain after release from the vector. Previous studies have provided evidence that a β-endorphin cationized albumin chimeric peptide undergoes absorptive-mediated endocytosis and exocytosis at the blood-brain barrier in vivo. Also, the brain contains the necessary disulfide reductases that are sufficiently active to cleave the chimeric peptide, which results in the release of free β-endorphin from its cationized albumin vector.[19] Cationized albumin has proved to be an effective blood-brain barrier transport vector for experimental studies in laboratory animals. The use of this vector in humans may be limited by the rapid renal uptake of cationized albumin, however.[17] While highly cationic proteins are known to be nephrotoxic,[20] recent studies have shown that mildly cationized homologous albumins may be administered repetitively to laboratory animals without measurable nephrotoxicity.[17] Nevertheless, it would be advantageous to develop vectors with a higher degree of brain specificity. Developing such vectors may invariably arise from a molecular biologic analysis of brain capillary-specific proteins. The production of monoclonal antibodies to brain capillary-specific proteins may prove to be brain transport vectors with a high degree of brain specificity.

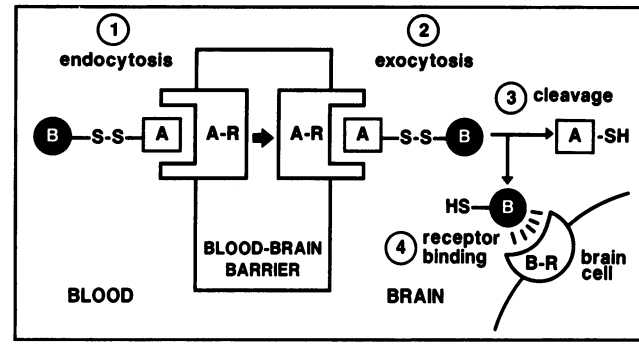

Figure 1.—The delivery of chimeric peptide through the blood-brain barrier is viewed as a process composed of 4 overall steps (see text). **A** is a transportable vector, for example, antitransferrin receptor monoclonal antibody; **B**, nontransportable (pharmacologically active) peptide, for example, β-endorphin; **A-R**, peptide A, for example, transferrin receptor; **B-R**, peptide B receptor (from Pardridge et al[19]; reproduced with permission from *Endocrinology*).

Blood-Brain Barrier Glucose Transporter, a Model Brain Capillary-Enriched Protein

RUBEN J. BOADO, PhD*: A vector with a relatively high degree of brain specificity is a monoclonal antibody to the transferrin receptor.[11] The transferrin receptor is a brain capillary-enriched protein,[21] that is, a protein found in high concentration in brain capillary endothelium relative to neurons or glial cells or to microvascular endothelium in peripheral tissues. Other brain capillary-enriched proteins are listed in Table 2.[1,21-33] When sections of animal or human brain are stained with antibodies to these proteins, the microvascular endothelium is markedly illuminated owing to the high concentration of these proteins in brain capillary endothelium. The brain capillary-enriched proteins, however, may also be localized in peripheral tissues. In contrast, brain capillary-specific proteins are thought to be expressed only in the brain capillary endothelium and not in peripheral tissues, and two brain capillary-specific proteins have been identified (Table 2).

The identification and molecular cloning of brain capillary-specific protein genes may lead to the development of monoclonal antibodies specific to these proteins. The molecular cloning of brain capillary-specific protein genes requires the development of brain capillary complementary DNA

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TABLE 2.—*Brain Capillary-Enriched Proteins and Brain Capillary-Specific Proteins**

Functional Class	*Protein*
Brain Capillary-Enriched Proteins	
Nutrient transporters	GLUT-1 glucose transporter
	Low Km NAA transporter
Peptide transporters	Transferrin receptor
	Insulin receptor
Drug transporters	P-glycoprotein (MDR gene product)
Vitamin transporters	Cellular retinol binding protein
Cholesterol transporters	Apolipoprotein A-I
Enzymes	γ-Glutamyltranspeptidase
	Aminopeptidase M
Phosphoproteins	50-55 KDa triplet
HLA antigens	Class I (endothelium)
	DR-antigen (pericyte, smooth muscle)
Surface antigens	CD36
	HT7
	140 KDa pericyte protein
	25 KDa triplet
Brain Capillary-Specific Proteins	
Surface antigens	45 and 53 KDa proteins

MDR = multidrug resistance, NAA = neutral amino acid

*From Pardridge,[1] compiled from data in references 21-33.

(cDNA) libraries. It is probably not possible to isolate cDNA clones corresponding to brain capillary-specific protein genes by screening cDNA libraries made from total brain. The volume of the brain capillary endothelial cell is only about 1 μl per gram of brain. There is only approximately 1 ml of endothelial cytoplasm in an entire human brain and only 1 μl of endothelial cytoplasm in an entire rat brain.[1] Therefore, less than 0.2% of the cDNA clones in a total brain library may be derived from reverse transcription of brain capillary-derived messenger RNA (mRNA). Consequently, it is necessary to isolate mRNA density from brain capillaries, which may be isolated from either laboratory animal or human autopsy brain (Figure 2).[34] Messenger RNA has been directly isolated from either bovine or pig brain capillaries, and blood-brain barrier cDNA libraries have been prepared in λgt10[32] or λgt11 vectors.[33] From these libraries, several brain capillary-enriched protein gene cDNAs have been cloned, including those for γ-glutamyl transpeptidase,[24] the GLUT-1 glucose transporter,[33,35] and apolipoprotein A-I.[32]

The blood-brain barrier GLUT-1 glucose transporter may be a brain capillary-enriched protein target for drug delivery because recent studies have shown the concentration of this protein in brain microvessels is particularly high, for example, 10 picomol per mg of protein.[36] Thus, the concentration of the GLUT-1 glucose transporter is approximately 100-fold more abundant than is the transferrin receptor.[37] In addition, the GLUT-1 glucose transporter protein is expressed selectively in brain microvascular endothelium with minimal, if any, expression in neurons or glial cells. Recent quantitative Western blotting, in situ hybridization, quantitative Northern blotting, and quantitative electron microscopic immunogold studies have all indicated that the GLUT-1 glucose transporter is selectively expressed in brain microvascular endothelium with no measurable expression in neurons or glial cells.[36,38,39] The GLUT-1 glucose transporter protein, however, is also expressed in peripheral tissues, including perivenous hepatocytes, renal collecting duct epithelium, peripheral nerve perineurium, syncytiotrophoblast of the placenta, placenta-fetal microvascular endothelium, and testicular microvascular endothelium.[40] Therefore, although the GLUT-1 transporter protein is specific to endothelium with respect to neurons or glial cells, it is also found in various epithelial and endothelial cells in peripheral tissues.

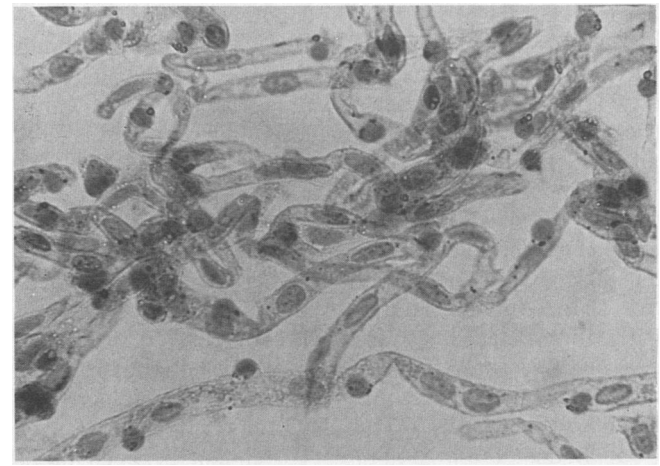

Figure 2.—A light micrograph shows capillaries isolated from a human brain at autopsy. The microvessels are comprised of endothelial cells and pericytes (in an approximate 3:1 ratio) encased in a common basement membrane (magnification × 500; from Pardridge et al[34]; reproduced with permission from *Journal of Neurochemistry*).

Biochemical Opening of the Blood-Brain Barrier—Leukotrienes

KEITH L. BLACK, MD*: Capillaries within primary tumors contain a blood-tumor barrier that impairs delivery of antitumor compounds to neoplastic cells within the brain.[41-43] The inability to delivery antineoplastic drugs across the blood-tumor barrier has led to experimental attempts to open the barrier.[44,45] One approach has used intracarotid infusion of mannitol, which causes a transient osmotic shrinkage of the brain capillary endothelial cells and disrupts the blood-brain barrier tight junctions in normal brain, as well as in brain tumors.[43,45-47] The osmotic barrier disruption approach nonspecifically increases the delivery of chemotherapeutic compounds and monoclonal antibodies to brain.[44] Osmotic disruption of the blood-brain barrier, however, results in only a modest increase in drug levels within the actual tumor, compared with the relatively large increase in drug levels in normal brain.[48] Therefore, this technique increases the exposure of the normal brain parenchyma to the deleterious effects of the chemotherapeutic compounds,[46] whereas suboptimal drug levels may actually reach the tumor. We recently described an experimental method that circumvents this problem and uses leukotrienes to selectively open the barrier within the tumor while leaving the normal blood-brain barrier intact.[49]

The rate-limiting step in leukotriene production is the 5-lipoxygenase enzyme. Brain lipids are rich in membrane-bound arachidonic acid. During a variety of metabolic events, arachidonic acid is released from cell membranes by phospholipase A2 of phospholipase C. Free arachidonic acid, once released, is either reincorporated into the cell membrane or oxidized by one of two pathways: the cyclooxygenase pathway to prostaglandins or the 5-lipoxygenase pathway to leukotrienes. The 5-lipoxygenase pathway—requiring molecular oxygen and calcium—generates the hydroxyleuko-

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triene B4 and the peptidoleukotrienes C4, D4, E4, and F4 via the epoxide intermediate leukotriene A4. The blood-brain barrier γ-glutamyl transpeptidase (Table 2) inactivates leukotriene C4 to leukotriene D4 and leukotriene E4 to leukotriene F4.[50] This enzyme is unique to brain capillaries as it is not present in systemic capillaries.[51]

Leukotrienes are quite potent in their ability to increase vascular permeability in systemic (noncerebral) capillaries.[52] Normal brain capillaries, however, resist the efforts of leukotrienes to increase their vascular permeability. In effect, normal brain capillaries seem to contain an "enzymatic barrier" that inactivates vasoactive compounds such as leukotriene C4. Injured brain capillaries or capillaries in brain tumors lose their "enzymatic barrier" and show increased vascular permeability after leukotriene C4 infusions.[49]

In human brain tumors, a correlation between brain edema and tissue levels of leukotriene C4 has been shown.[53] An index of peritumoral edema was estimated from preoperative contrast-enhanced computed tomographic scans. There was a significant correlation between edema and the log leukotriene C4 levels, with $P < .003$ in all patients. Metastatic tumors had the highest leukotriene C4 level at 13.8 ± 8.5 pg per mg of tissue (mean ± standard error) and the highest index of edema at 5.7 ± 1.8. The mean leukotriene C4 level in gliomas was 6.2 ± 2.3 pg per mg of tissue and the edema index was 2.1 ± 0.6. There was no edema, no tumor in the temporal lobes removed for seizure, and their level of leukotriene C4 was 0.4 ± 0.1 pg per mg of tissue. These data show that the formation of leukotriene C4 is stimulated by intracranial tumors.

In experimental brain tumors in rats (C6 glioma, RG-2 glioma, and Walker 256 metastatic tumor) γ-glutamyl transpeptidase could not be shown within tumor capillaries.[49] Because brain tumors stimulate leukotriene C4 formation, and tumor capillaries—unlike normal brain capillaries—lack γ-glutamyl transpeptidase to inactivate leukotriene C4, leukotrienes could play an important role in the development of vasogenic edema surrounding brain tumors and in increasing capillary permeability within tumors.

It is important for delivery of antitumor compounds to have the ability to further open the capillary barrier within tumors. Recently intracarotid infusions of leukotriene C4 were shown to selectively open the blood-tumor barrier in rats with RG-2 gliomas.[49] Blood-brain and blood-tumor permeability was determined by quantitative autoradiography using ^{14}C aminoisobutyric acid. Leukotriene C4 (total dose, 4 μg) infused into the carotid artery ipsilateral to the tumor increased twofold the unidirectional transfer constant for permeability within the tumor, whereas no effect on permeability was seen in normal brain. No γ-glutamyl transpeptidase activity was seen in tumor capillaries, in contrast to high γ-glutamyl transpeptidase in normal brain capillaries. These findings support the hypothesis that normal brain capillaries resist the vasogenic effects of leukotriene C4, whereas leukotriene C4 may increase permeability in tumor capillaries.

Loss of γ-glutamyl transpeptidase is not, however, limited in brain to tumor capillaries. γ-Glutamyl transpeptidase activity is also decreased in capillaries in ischemic tissue at 48 and 72 hours after middle cerebral artery occlusion in rats.[54] In comparison, high γ-glutamyl transpeptidase in normal brain capillaries and moderate γ-glutamyl transpeptidase in capillaries in ischemic tissue 24 hours after middle cerebral artery occlusion was reported. At 72 hours after middle cerebral ischemia, the infusion of leukotriene C4 (total dose, 4 μg) into the carotid artery ipsilateral to the ischemia selectively increased blood-brain barrier permeability approximately threefold within core ischemic tissue and tissue adjacent to the ischemic core.[54] No effect on blood-brain barrier permeability was seen within nonischemic brain tissue or in ischemic tissue after only 24 hours of ischemia.

Acivicin is an inhibitor of γ-glutamyl transpeptidase.[54] When rats are pretreated with acivicin, the ability of leukotriene C4 to increase blood-brain barrier permeability in ischemic brain even at 24 hours is significantly enhanced. At 24 hours, when only a moderate reduction in γ-glutamyl transpeptidase has occurred, further blocking of γ-glutamyl transpeptidase activity with acivicin results in a significant enhancement of the ability of leukotriene C4 to open the blood-brain barrier.[54]

These experiments show that brain capillaries contain an "enzymatic barrier" that inactivates vasoactive compounds, like leukotrienes, which increase blood-brain barrier permeability. In some pathologic conditions—that is, brain tumors, ischemia—this "enzymatic barrier" is lost. One component of the enzymatic barrier seems to be γ-glutamyl transpeptidase. Its loss in tumor capillaries and in ischemia permits leukotriene C4 to increase vascular permeability in these conditions and produce vasogenic edema. Because leukotriene C4 production is significantly increased in brain tumors, leukotrienes may play an important role in edema surrounding brain tumors. Clinically important, the selective vulnerability of tumor capillaries to leukotrienes can be used therapeutically. Because tumor capillaries in brain tumors form a barrier to many chemotherapeutic compounds and monoclonal antibodies, leukotriene C4 may be used to open selectively the barrier within tumors while leaving the normal blood-brain barrier intact. One important advantage of selective barrier opening would be protecting normal brain tissue from the potentially deleterious effects of antineoplastic compounds.

Biochemical opening of the blood-brain barrier by vasoactive substances such as leukotrienes may involve cell-to-cell interactions at the cerebral microcirculation. The latter is comprised of at least four cell types: endothelium, which constitutes the permeability barrier; astrocyte foot processes, which invest more than 95% of the surface of the brain capillary; pericytes, which have contractile and phagocytic functions at the microcirculation; and nerve endings, which directly innervate brain microvascular endothelium.[1] To understand the mechanisms of cell-to-cell interactions at the brain microcirculation, in vitro tissue culture models have been established to investigate the cell biology of endothelium-astrocyte interactions.

Cell Biology of Brain Endothelium and Astrocyte Interactions

PASQUALE A. CANCILLA, MD*: Our laboratory has used in vivo and in vitro systems to explore the complex interactions that occur between endothelial cells and astrocytes and that lead to the development of properties associated with the blood-brain barrier. The in vivo system most often used by us has been the localized application of liquid nitrogen to the intact skull of the mouse to produce a discrete freeze injury.[55]

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This area of injury undergoes a reproducible series of changes including necrosis, monocyte-macrophage infiltration, endothelial proliferation and neovascularization, and astrocyte proliferation and migration that lead to restitution of endothelial-astrocyte relations and restoration of the blood-brain barrier. Our studies used ^{3}H-thymidine as a marker for the initiation of DNA synthesis in endothelial cells and astrocytes at intervals after the injury.[56,57] Both endothelial cells and astrocytes are normally quiescent cells that quickly return to the mitotic pool in response to injury. This coincides with the migration of monocyte-macrophage into the area of injury from the circulation. Interruption of the monocyte-macrophage response reduces the endothelial response to approximately 10%. Thus, factors derived from the monocyte-macrophage are operational in the endothelial response. Cytokines may also be involved in restoring the endothelium of the injured vessels and in neovascularization because in-vitro studies have shown a chemotactic effect of monocyte-macrophage on endothelium.[58]

The astrocytes adjacent to the area of injury undergo a similar ^{3}H-thymidine incorporation after an injury, but the maximum response is of lower magnitude (10%) and occurs later—day 5 compared with day 3 to 4 after injury—than the endothelial cell response. In vitro studies have shown that cytokines derived from monocyte-macrophage may be operational in this response and that endothelial cells may liberate chemotactic factors, which attract astrocytes from the uninjured brain adjacent to the injured area and to the new vessels.[58] Studies using the freeze-injury model have shown that endothelial cells in vessels adjacent to the injury initially have the enzyme γ-glutamyl transpeptidase, but within five to seven days of the injury, the enzyme is no longer found in the endothelium.[57] This corresponds to the time when the endothelial cells migrate into the area of injury as part of the process of neovascularization and correlates with the loss of contact of the astrocyte with the endothelium.[56] When the astrocyte reestablishes contact with the endothelial cell, γ-glutamyl transpeptidase is found once again in the endothelium.[57] This supports the in vivo findings that interaction of the astrocyte with the endothelial cell is necessary for the induction and maintenance of γ-glutamyl transpeptidase expression and that a factor liberated by the astrocyte is responsible for this effect.[59,60]

Endothelial cells influence responses in the astrocyte. Media conditioned by cerebral endothelial cells contain a factor or factors that stimulate the incorporation of ^{3}H-thymidine by the astrocyte.[61] This factor is a peptide of greater than 50,000 molecular weight that is destroyed by protease digestion and is different from other known growth factors. Rather than stimulating ^{3}H-thymidine incorporation in endothelial cells through an autocrine effect, the factor does not affect endothelial cells.

Dr Takashi Minakawa, in our laboratory, has used his model of developing vascular-like structures in vitro into a model replicating the relation of the vascular endothelium and astrocytes.[62] The system used gelatin-coated slides in chambers as a base for developing branching tube-like structures when coated with vitrogen. Astrocytes or pericytes added to the surface of the vitrogen migrate through this layer and develop contacts with the endothelial structures. Astrocytes and pericytes respond differently. By 24 hours after the random addition of the pericytes to the system, 100% of the cells are in contact with the endothelium. Astrocytes are slower to respond because 10% are in contact by 5 hours, 75% are associated with the vessels at 24 hours, and 93% by 6 days.

A chemotactic assay has been developed using several features from this system.[62] Use of it has shown that these vascular-like structures, but not monolayer cultures of endothelium, release a chemotactic factor involved in the attraction of the astrocyte to the endothelium. This factor is liberated from the better-differentiated cells forming the vascular tubes and suggests that the response is coordinated and may serve to attract the astrocyte to the endothelium, as was suggested by the in vivo model.

The studies described in this presentation represent some, but not all, of the types of studies that have been done to show that the cell-to-cell interaction between endothelium and astrocyte is important in establishing and maintaining the blood-brain barrier.[63-68] Many investigators are interested in this area, so we may soon see important and detailed reports describing not only the cell biology but also the molecular mechanisms of these complex and fascinating relations.

Ongoing basic research into the molecular and cell biology of the blood-brain barrier is expanding further the definition of brain capillary-specific proteins and the molecular mechanisms involved in paracrine cellular interactions at the level of the cerebral microcirculation. This research invariably provides insights into the pathogenesis of brain diseases.[63] Basic research in blood-brain barrier molecular and cell biology has also led to new approaches to brain drug delivery. As molecular and neurobiologic research further defines the mechanisms of disorders of the brain, new therapies may emerge. An essential component for developing neuropharmaceuticals of the future will be integrating these with the emerging paradigms of blood-brain barrier drug delivery.

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